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APPLICATION NUMBER: *60/582,221*
FILING DATE: *June 24, 2004*

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PTO/SB/16 (04-04)
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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.	

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Herve	Bercovier	Jerusalem ISRAEL

Additional inventors are being named on the 2 of 2 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

A Pharmaceutical Composition and Method for Treating a Disease and a Diagnostic Tool Therefor

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 15
☑ Drawing(s) *Number of Sheets* 6
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☑ Other (specify) Postcard

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[Page 1 of 2]

Respectfully submitted,

SIGNATURE

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Date June 24, 2004

REGISTRATION NO. 41,827 *(if appropriate)*

Docket Number: 26224 PRO

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Docket Number	26224 PRO

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Number 2 of 2

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Attorney Docket No. 26224PRO

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

BERCOVIER et al.

Serial No. NOT YET ASSIGNED

Filed: June 24, 2004

For: **A PHARMACEUTICAL COMPOSITION AND METHOD FOR TREATING A DISEASE AND A DIAGNOSTIC TOOL THEREFOR**
(Provisional Application)

TRANSMITTAL LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Submitted herewith for filing in the U.S. Patent and Trademark Office is the following **PROVISIONAL APPLICATION:**

(1) Transmittal Letter
(2) Cover sheet for filing **Provisional Application**
(3) 21 page Provisional Application consisting of:
13 pages Textual Specification,
2 pages of references,
6 sheets of Drawings;
(4) Check No. 2125 $ 80.00 for filing fee as a small entity;
(5) Postcard for early notification of serial number.

The Commissioner is hereby authorized to charge any deficiency or credit any excess to Deposit Account No. 14-0112.

Respectfully submitted,
NATH & ASSOCIATES PLLC

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A PHARMACEUTICAL COMPOSITION AND METHOD FOR TREATING A DISEASE AND A DIAGNOSTIC TOOL THEREFOR

FIELD OF THE INVENTION

This invention relates to the diagnosis and treatment of diseases and disorders with a unique amino acid molecule, and in particular, to treatment of tuberculosis.

BACKGROUND OF THE INVENTION

Tuberculosis (TB) is mostly a pulmonary lung disease caused by *Mycobacterium tuberculosis* (*Mtb*). This organism is a slow-growing bacillus that is transmitted by the respiratory route. Soon after infection, the bacilli are phagocytosed by alveolar macrophages and survive within early phagosomes. Innate immune responses directed by macrophages predominate early in infection. Subsequent recruitment of dendritic cells leads to cell-mediated responses involving $CD4^+$ and $CD8^+$ T cells and eventual granuloma formation. Among infectious diseases, tuberculosis remains the second leading killer of adults in the world, with more than 2 million TB-related deaths each year (CDC 2004). The vast majority of immunocompetent individuals are able to contain, but not eliminate, the pathogen in pulmonary granulomas, leading to latent tuberculosis infection a small minority of cases, through unclear mechanisms, persistent bacilli can reactivate to form disease many years to decades after initial infection.

Virulent *Mycobacterium* cell wall-associated lipids

A major cell surface component of *Mtb* and other virulent *mycobacterium* sp. is the polysaccharide manosylated lipoarabinomannan (manLAM). ManLAM

is a phosphatidylinositol-anchored lipoglycan composed of a mannan core with oligoarabinosyl-containing side-chains with diverse biological activities. This polysaccharide accounts for up to 5 mg g -1 bacterial weight. ManLAM structure differs according to mycobacterial species, and three general classes of manLAM have been described: (i) ManLAM, from the virulent strains Erdman and H37Rv and the avirulent strains H37Ra and BCG [Chatterjee, D., et al. J Biol Chem 267: 6234-6239 (1992); Prinzis, S., et al. J Gen Microbiol 139: 2649-2658 (1993)], which is characterized by extensive mannose capping of the arabinan termini; (ii) phospho-myo-inositol-capped manLAM (PILAM), found in the rapidly growing mycobacteria M. smegmatis and M. fortuitum [Nigou, J. Biochimie 85: 153-166 (2003)]; and (iii) AraLAM, which was described in the rapidly growing M. chelonae and lacks mannosylation in its arabinan termini [Guerardel, Y., et al. J Biol Chem 277: 30635-30648 (2002)]. Although there is significant heterogeneity between manLAM molecules with respect to glycosylation and acylation [Nigou et al. (2003) ibid.], differences in biological activity between the major classes of manLAM have been attributed primarily to the heavy mannose capping of ManLAM or to differences in the phosphatidylinositol anchor [Chatterjee et al. (1992). Ibid.]

In several publications it has been shown that sera of TB patients contain higher levels of anti-manLAM antibodies then that of healthy individuals [Hetland G, et al. Clin Diagn Lab Immunol. 5(2):211-8 (1998)]. Consequently it has been suggested to be one of the candidate antigens for rapid diagnosis of TB [Antunes A, et al. Res Microbiol. 153(5):301-5 (2002)].

DESCRIPTION OF THE INVENTION

In the description herein, the following abbreviations are used, all acceptable in the art:

Mtb - Mycobacterium tuberculosis

TB - Tuberculosis

TBS - tris buffer saline

PBS – phosphate buffer saline

manLAM- Mannosilated Lipoarabinomannan

Ab- Antibodies

mAb – Monoclonal Ab

Ag- Antigen

Hr -Hours

ELISA- Enzyme-linked immunosorbent assay

RT- Room temperature

Aa –Amino acid

BSA- Bovine Sera Albumin

KLH - keyhole limpet hemocyanin

IFA - incomplete freund's adjuvant

DDA - dimethyldioctadecylammonium bromide

SPF - Specific pathogen-free

IP- intra peritoneal

SC – subcutaneous

FACS - flow cytometry

The present invention is based on the finding of a unique peptide which acts as a manLAM mimotope. This peptide, having the amino acid sequence: **ISLTEWSMWYRH** (SEQ ID NO:1) was shown to have the following characteristics:

1. It binds competitively to a manLAM binding monoclonal antibody (mAb).

2. When vaccinating with the peptide cross-reactive anti manLAM antibodies are elicited.

3. Sera tested from TB patients (16/16) and Mtb infected mice (10/10) has significant levels of antibodies that bind the mimotope peptide.

Thus, according to one of its aspects, the present invention provides a pharmaceutical composition (e.g. a vaccine) comprising a pharmaceutically

acceptable carrier and as an active ingredient an amino acid molecule comprising the sequence selected from:

(a) the amino acid sequence **ISLTEWSMWYRH (SEQ ID NO:1)**

(b) a biologically functional analog of the amino acid sequence of (a).

The "***Amino acid sequence***" according to the invention is a sequence composed of any one of the 20 naturally appearing amino acids, amino acids which have been chemically modified or synthetic amino acids

The term "***biologically functional analog***" refers to any peptide comprising a region which when optimally aligned with the sequence of (a) has at least 90% homology with the latter.

"***Optimally alignment***" is defined as an alignment giving the highest percent identity score

The variation in amino acid sequence between the homologue and the sequence of SEQ ID NO:1 or a fragment thereof, arises from the addition, deletion, or substitution or chemical modification of one or more amino acids of the sequence of SEQ ID NO:1. Where the homologue contains a substitution, the substitution is preferably a conservative one.

"***Conservative substitution***" as used herein refers to the substitution of an amino acid in one class by an amino acid of the same class, where a class is defined by common physicochemical amino acid side chain properties and high substitution frequencies in homologous proteins found in nature, as determined, for example, by a standard Dayhoff frequency exchange matrix or BLOSUM matrix. Six general classes of amino acid side chains have been categorized and include: Class I (Cys); Class 11 (Ser, Thr, Pro, Ala, Gly); Class III (Asn, Asp, Gln, Glu); Class IV (His, Arg, Lys); Class V (Ile, Leu, Val, Met); and Class VI (Phe, Tyr, Z= I Trp). For example, substitution of an Asp for another class III residue such as Asn, Gln, or Glu, is a conservative substitution.

"***Non-conservative substitution***" refers to the substitution of an amino acid in one class with an amino acid from another class; for example, substitution

of an Ala. a class 11 residue, with a class III residue such as Asp, Asn, Glu. or Gln.

"***Deletion***" is a change in amino acid sequence in which one or more amino acid residues is absent as compared to the original sequence having designated as SEQ ID NO:1.

"***Insertion***" or "***addition***" as used herein denotes a change in an amino acid sequence which has resulted in the addition of one or more amino acid residues as compared to the original sequence having designated as SEQ ID NO:1

"***Substitution***" as used herein denotes replacement of one or more amino acids by different amino acids as compared to the original sequence having designated as SEQ ID NO:1. The substitution may be conservative or non-conservative, however, is preferably a conservative one.

The term ***"pharmaceutically acceptable carrier"*** refers to any vehicle, adjuvant, excipient, diluent, which is known in the field of pharmacology for administration to a human subject and is approved for such administration. The choice of carrier will be determined by the particular active agent, for example, its dissolution in that specific carrier (hydrophilic/hydrophobic), as well as by other criteria such as the mode of administration. One specific example is a vaccine comprising the peptide as defined and a pharmaceutically acceptable carrier suitable for injection.

The pharmaceutical composition according to the invention is preferably for the treatment or prevention of a disease, disorder or pathological condition.

According to one preferred aspect of the invention, the disease is and infectious disease and preferably, a microbactarial caused disease. Most preferably, the disease is Tuberculosis (TB).

The term "***treatment or prevention***" as used herein refers to the administration of a therapeutic amount of the active agent according to the

invention which is effective in one of the following: ameliorating undesired symptoms associated with a disease, disorder, or pathological condition. For example, in case of TB the symptoms may be coughing, dyspnea, pleural effusion; effective in preventing the manifestation of such symptoms before they occur; effective in slowing down the progression of a disease or disorder; effective in slowing down the deterioration of the disease (for example, in case of TB, to prevent the development of pleural TB or tuberculous empyema and bronchopleural fistula) in restricting the spreading of psoriasis to healthy region); effective to prolong the time period onset of remission period; effective in slowing down the irreversible damage caused in a progressive chronic stage of a disorder; effective to delay the onset of said progressive; effective to lessen the severity or cure the disease or disorder; effective to improve survival rates of individuals infected with the disease, or effective to prevent the disease form occurring altogether (for example in an individual generally prone to the disease) or a combination of two or more of the above.

According to another aspect, the invention concerns a method of treatment of a subject in need, the method comprises providing said subject with a therapeutically effective amount of an amino acid molecule comprising a sequence selected from:

(a) the amino acid sequence **ISLTEWSMWYRH (SEQ ID NO:1)**

(b) a biologically functional analog of the amino acid sequence of (a).

According to yet another aspect of the invention there is provided the use of an amino acid molecule for the preparation of a pharmaceutical composition, the amino acid molecule comprising a sequence selected from:

(a) the amino acid sequence **ISLTEWSMWYRH (SEQ ID NO:1)**

(b) a biologically functional analog of the amino acid sequence of (a).

Yet further, the present invention concerns a diagnostic kit comprising a composition comprising:

(i) an amino acid molecule comprising the sequence selected from:

(a) the amino acid sequence **ISLTEWSMWYRH (SEQ ID NO:1)**

(b) a biologically functional analog of the amino acid sequence of (a); and

(ii) instructions for use of said composition in diagnosis of a disease in a subject susceptible of carrying the disease or in diagnosis the severity of a disease in a subject diagnosed for carrying the disease.

The kit of the invention may be used in various ways, e.g. in combination with ELISA or in combination a solid phase technology such as dot blot where the peptide is bound to a solid phase and used to analyze sera from subject suspected of carrying a disease, such as TB. The Ab titers are then determined by a secondary anti-human antibody.

BRIEF DESCRIPTION OF THE DRAWINGS

In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

Fig. 1 is a bar graph showing the ability of CS40 linear peptide recombinant phage to induce production of cross reactive Ab that bind manLAM in mice immunized with CS40 linear peptide recombinant phage, as compared to mice vaccinated with control phage or control naïve mice.

Fig. 2 is a graph showing the competitive binding of CS40 linear peptide to manLAM antibodies.

Fig. 3 is a bar graph showing level of titer of anti CS40 linear peptide when being conjugated to keyhole limpet hemocyanin (KLH) via Cystein and emulsified in the MPL® + TDM Adjuvant system, as compared to pre-immune or naïve mice, or mice treated with KLH-TDM.

Fig. 4 is a bar graph showing level of titer of anti CS40 linear peptide after 30 days or 3 months as compared to naïve mice.

Fig. 5 is a bar graph showing lever of titers of anti manLAM and anti CS40 linear peptide in sera of tuberculosis patients (Mtb patients) compared to healthy subjects (negative control) and vaccinated subjects (vaccinated).

Fig. 6A-6B are FACS analysis diagrams showing the binding of CS40 linear peptide FITC conjugated to 50% of cells of the RAW 264.7 cell line (Figure 6A), and wherein the presence of 0.125 D-mannose the binding of the FITC conjugated peptide is reduced to 18% (Figure 6B).

Fig. 7A-E are FACS analysis diagrams showing the anti apoptotic effect on LPS activated RAW 264.7 cell line.

Fig. 8A-C are bar graphs showing NO cytokine secretion in LPS activated RAW 264.7 effected by the CS40 linear peptide.

DESCRIPTION OF THE SPECIFIC EXAMPLES

MATERIALS AND METHODS

Biopanning

Biopanning was performed based on Smith and Scott 1993, briefly 6 well polystyrene plates were coated with mAbs 5μg/well in Tris buffer saline (TBS), and then blocked with TBS/0.25% gelatin for 2hr RT. 10^{11} phages in TBS/0.25% gelatin were bound to the Ab at 4°C for 16hr the unbound phages were washed in 4 times in TBS and the bound phages were eluted with glycine-HCL/BSA pH 2.2 for 10m and neutralized with tris buffer pH 9.1.

Determination of clones binding the mAbs was performed by dot blot and manLAM was used as a positive binding control. Second and third rounds of biopanning were performed if no binding clones were found.

Anti manLAM antibodies and manosylated lipoarabinomannan (manLAM) were provided By Drs. Patrick Brennan and John Belsile (Colorado State University, Fort Collins, CO; National Institute of Health grant AI-75320)

Enzyme-linked immunosorbent assay (ELISA)

In general the assay was performed by coating 96 well Nunc Immuno™ maxisorp plates with 50μl of the relevant Ag. The plates were washed twice in

phosphate buffer saline (PBS) and blocked with 1% BSA in PBS then washed twice and 50µl/well of mAb/Sera were added and incubated for 1 hour at 37°C. After washing, alkaline phosphatase-labeled goat anti-mouse immunoglobulin (Sigma Cat# A-1902) was added. Following a 90-min incubation at 37°C, *p*-nitrophenyl phosphate (KPL Cat # 50-80-00) was added to the plates and optical density was measured at 405 nm (Shin et al 2001).

Concentration of Ab, sera and Ags

For direct phage ELISA wells were coated with 10^9 phage particles per well in TBS.

When peptide ELISA was performed wells were coated with 10 µg peptide per well in PBS. manLAM (50 µg/ml) was used to coat wells of immulon® 4HBX plates.

For analysis of mouse sera the sera were diluted 1/50 in PBS, mAb was used at 5µg/well.

Assays using human sera were performed according to Navoa et al 2003.

Competitive ELISA was performed according to Kaur 2002.

Peptide synthesis

Peptide sequence ISLTEWSMWYRH was synthesized by solid phase by Prof. Matti Fridkin at the Weitzman institute Rehovot, Israel, and by Mrs Lilian Glazer at the Hadassah Medical School, Jerusalem, Israel.

Immunization

For immunization, peptide was conjugated to keyhole limpet hemocyanin (KLH) via cysteine at the peptide amino terminus. 2mg of peptide were conjugated to the KLH according to manufactures instructions (Pierce Cat# 77606).

Specific pathogen-free (SPF) female BALB/c mice, 5–8 weeks old, were injected with 5µg or 50 µg conjugated peptide intra peritoneal (ip) or subcutaneously (sc) and boosted with the same dose three weeks from immunization. Various

adjuvants were tested as following; peptide conjugate was emulsified in MPL® + TDM Adjuvant system (sigma Cat. # M-6536), in incomplete freund's adjuvant (IFA) (sigma Cat # F-5506) both according to manufactures instruction and in dimethyldioctadecylammonium bromide (DDA) (Fluka, Switzerland. Control groups were immunized with the adjuvant only.

FITC binding and flow cytometry (FACS) analysis 50μg of peptide were conjugated to FITC as described by Sethi et al. [Sethi et al. Cell Motil Cytoskeleton. 52(4):231-41 (2002)]. Cell line RAW 264.7 American Type Culture Collection (ATCC) was grown in complete Dulbecco's modified Eagle's medium (Biological industries, Beit Haemek) to a 80% confluent culture. For binding assay cells were washed three times in cold PBS and harvested from flask. Cells were then incubated with FITC conjugated peptide for 15 minutes at 37°C centrifuged at 3000rpm 4°C washed 3 time in cold PBS. The fluorescence levels were determined by FACS analysis. Competitive binding of mannose was performed according to Nguyem and Hildreth [Nguyen DG, Hildreth JE. Eur J Immunol. 33(2):483-93 (2003)].

RESULTS

Finding manLAM mimotope and specific binding determination

In a search for a manLAM mimotope, random phage display libraries with mAbs were screened. As a result, a linear 12 amino acid peptide that mimics manLAM (mimotope) was found. In particular, screening was performed using mAb CS40, and a clone presenting a linear 12 amino acid sequence ISLTEWSMWYRH was found.

Testing the binding of the positive phage clone to the CS40 antibody was performed by Dot-Blot and ELISA essays. To further test the recombinant phages' affinity to the antibody, competitive ELISA was performed. The phage clone showed the ability to compete with the binding of the Ab to the manLAM.

Immunization with phage clone CS40 linear mimotope

To test the ability of CS40 linear mimotope recombinant phage to induce production of cross reactive Ab that bind manLAM, 5 mice were immunized with 10^{12} phages and boosted two weeks after first injection. The sera were pooled and tested by ELISA for anti manLAM IgG Ab. As control, sera from naïve mice as well as mice vaccinated with control phage were tested. The mice vaccinated with the CS40 linear mimotope had specific cross reactive anti manLAM IgG Antibodies (Figure 1).

Testing the binding properties of the peptide mimotope

A peptide corresponding to the CS40 mimotope phage clone was synthesized. The binding of the synthetic peptide to CS40 mAb was tested by direct ELISA and by competitive ELISA. It was shown that the synthetic peptide mimics a manLAM epitope; the Ab bound the peptide in direct ELISA assay and showed an ability to compete with the binding of the Ab to manLAM (figure 2).

Immunization with the peptide mimotope

To test if the peptide elicits the production of Ab cross reactive to manLAM Balb/c mice were vaccinated with the synthetic CS40 linear peptide mimotope in various routs of administration and adjuvants.

It was found that the peptide preparation that elicited cross reactive Ab was the mimotope conjugated to keyhole limpet hemocyanin (KLH) via Cystein and emulsified in the MPL® + TDM Adjuvant system. Specifically, each mouse received 50 μg of peptide per injection of this formula. The results shown in Figure 3 suggest that the peptide mimotope is a potential vaccine candidate for tuberculosis.

***Mtb* Infected mice produce anti peptide mimotope Ab**

Thirty days (n=6) and three months (n=4) after *Mtb* infection Balb/c mice were tested for IgG anti manLAM and anti CS40 linear mimotope Abs, and were compared to naive mice (n=6).

The *Mtb* infected mice showed both anti manLAM and anti CS40 linear mimotope Ab. The Ab levels of the anti manLAM and anti mimotope in the mice were similar; however, the standard deviation between the mice in the case of mimotope was lower. Figure 4 shows the Ab level of anti-manLAM and anti-mimotope in the three tested groups (30 days, 3 months and naïve mice)

IgG anti CS40 linear peptide antibodies in human tuberculosis patients

Significantly higher titers of anti manLAM and anti CS40 linear peptide exist in sera of tuberculosis patients (n=16) compared to healthy people (n=36). As in the case of mice sera the human Ab levels of the anti manLAM and anti mimotope are similar but the variability (standard deviation) in the case of the mimotope was lower. This gives indication that the peptide can be a more reliable diagnostic tool then manLAM. Sera from BCG vaccinated people (n=10) were tested and the Ab titers shown were significantly lower then in TB patients and higher then in the healthy non vaccinated patients (Figure 5). This may thus serve as a tool to differentiate vaccinated between sick and healthy subjects.

CS40 linear peptides binds macrophage cell line RAW 264.7

CS40 linear peptide FITC conjugate was found to bind to Macrophage cell line RAW 264.7(Figure 6A). This binding was inhibited by D-mannose (Figure 6B).

Anti apoptotic effect on LPS activated RAW 264.7 cell line

It has been shown previously by others that ManLAM inhibits apoptosis in macrophages and LPS induced apoptosis in dendritic cells. The mimotopes' effects on apoptosis were tested. The mimotope peptide showed the ability to enhance LPS + INFγ induced apoptosis at the concentration of 0.3 μg/ml and to inhibit it in higher doses (0.5-100 μg/ml) in a dose dependent matter in RAW 264.7 cell line (Figs. 2A-E). The peptide itself does not induce or suppress apoptosis on non activated cells (data not shown).

NO and cytokine secretion in LPS activated RAW 264.7 effected by the CS40 linear peptide.

The effects of ManLAM on NO and cytokine secretion in macrophage and dendritic cells has been demonstarted. It has been shown that ManLAM has an inhibitory effect on cells activated by LPS. These effects of the CS40 linear peptide on the LPS activated macrophages were tested and compared to the ManLAM effects on the cells.

These effects were tested both after long and after short term treatment. Short term effect of both the ManLAM and the CS40 linear peptide was detected in the secretion of TNFα and INFγ. Higher levels of these cytokines are detected in cells were the ManLAM and the CS40 linear peptide were added to the LPS treated cells with very similar amounts when comparing the peptide the mycobacterial polysaccharide. After long term treatment the higher amounts of TNFα are detected with both treatments. Comparing the INFγ a difference is observed and while with peptide the levels remain high, with the ManLAM levels seem to be as with LPS only (Figs. 8A-B).

NO and IL12 levels produced by peptide treatment did not seem different than the LPS only treated cells after short term treatment. IL10 secretion was not effected by the CS40 linear peptide neither after long or short treatment. After long term incubation levels of IL12 that were detected are higher in the cells were the peptide was added, as shown in the Figure.

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Figure 1:

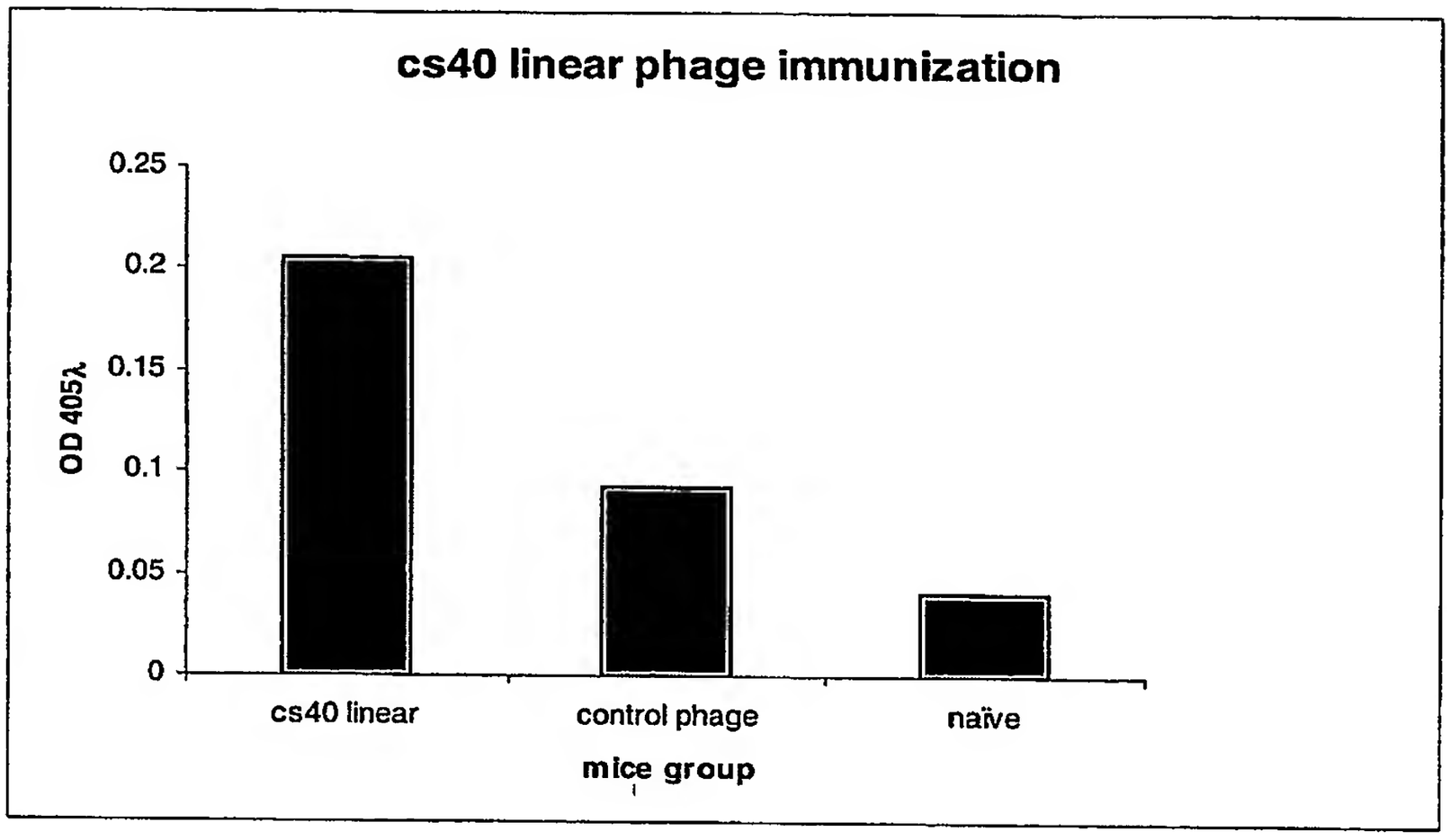
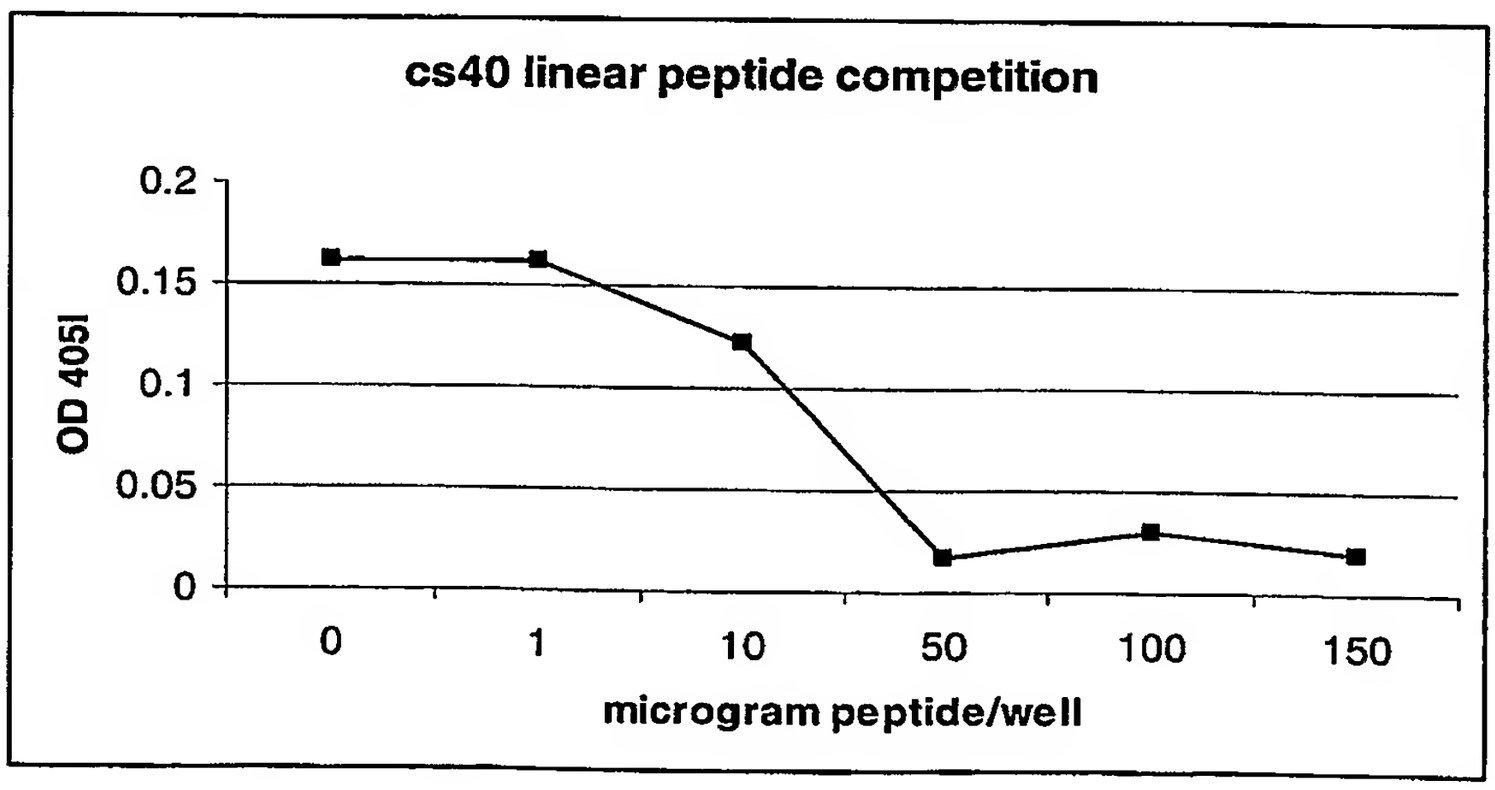


Figure 2:



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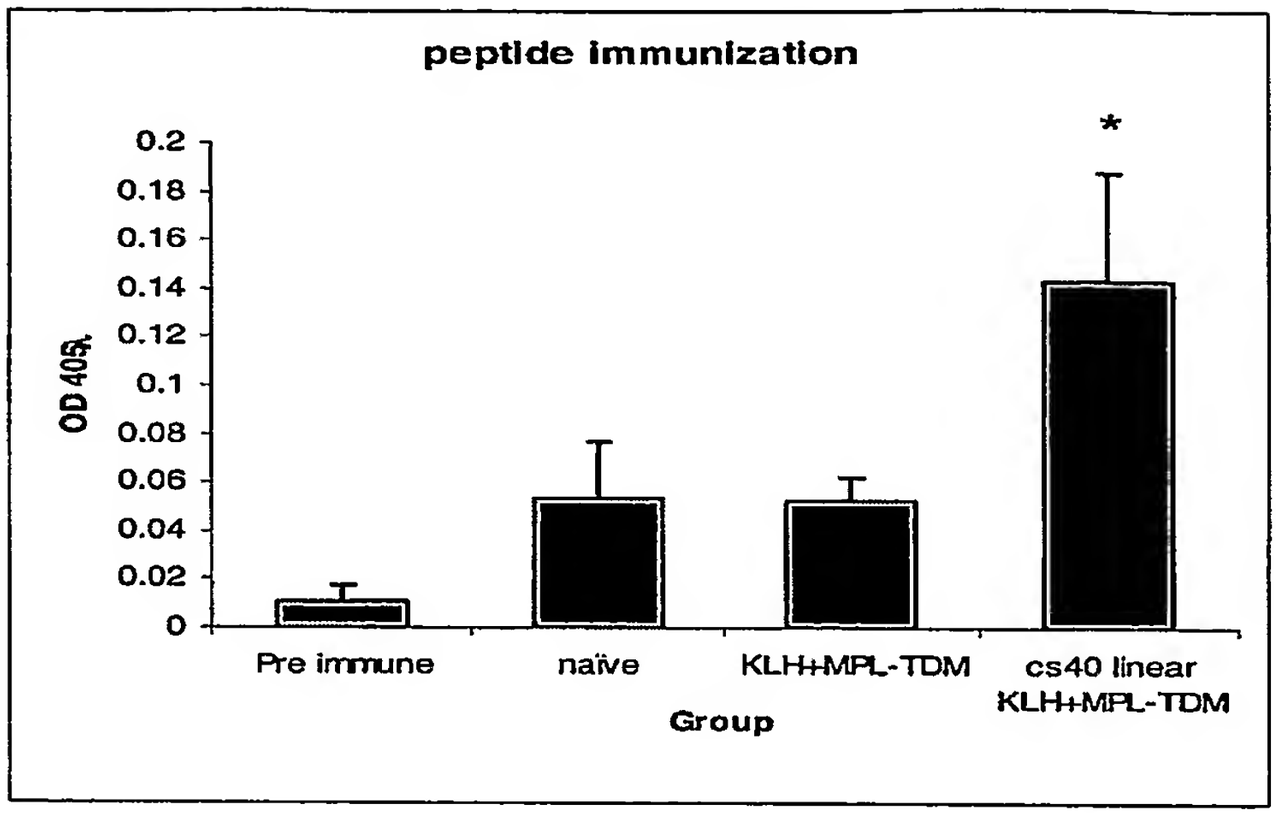

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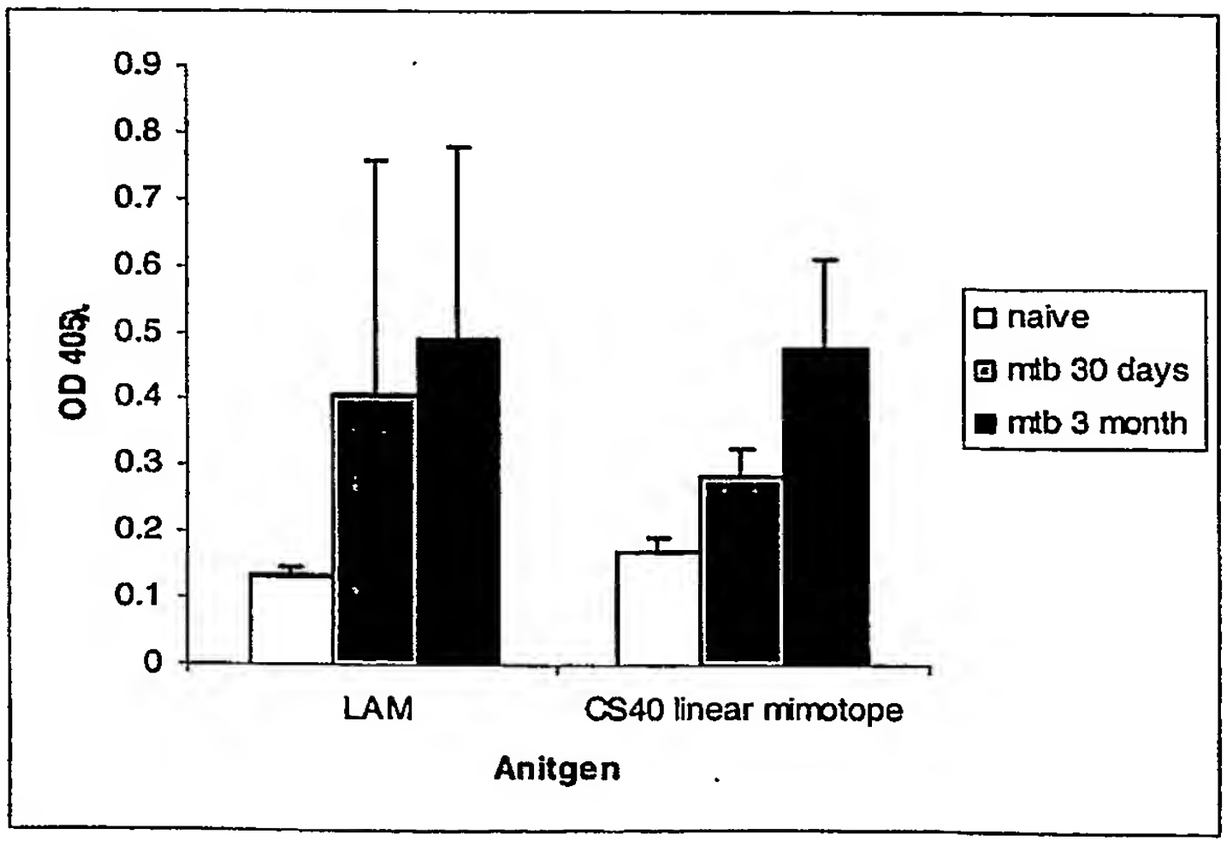

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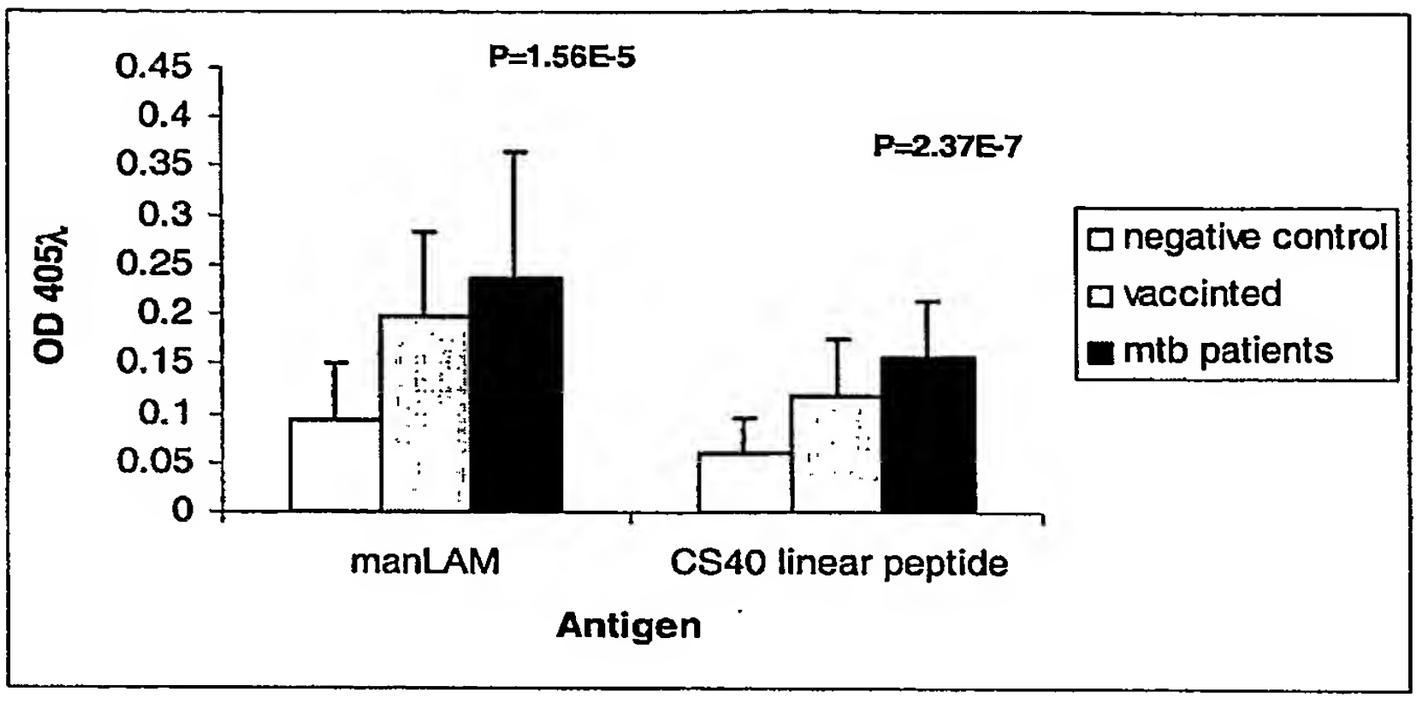


Figure 6A

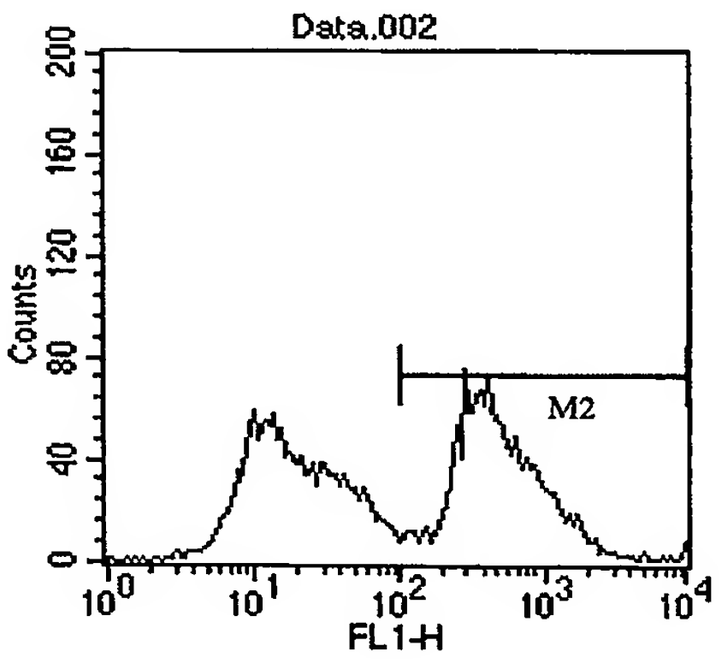


Figure 6B

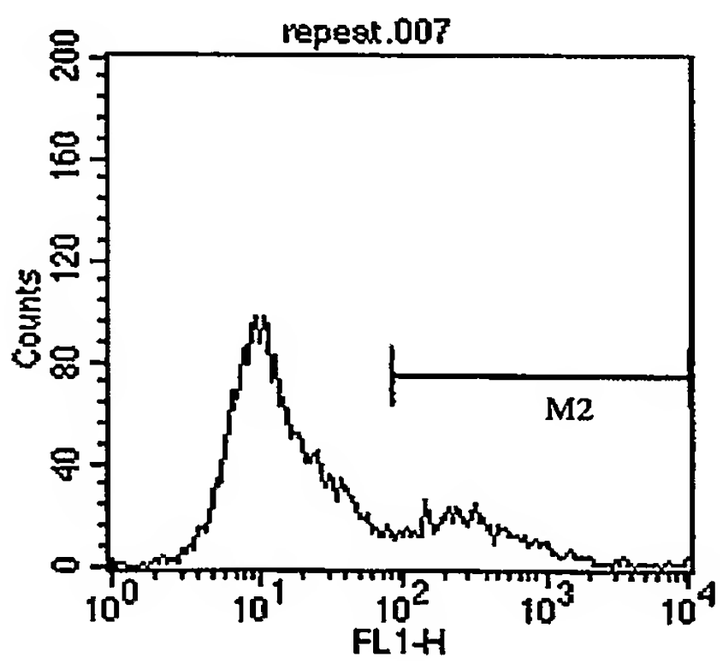


Figure 7A

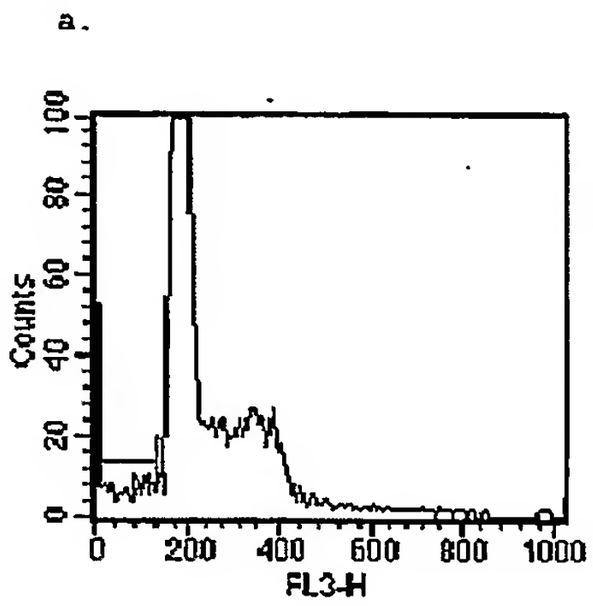


Figure 7B

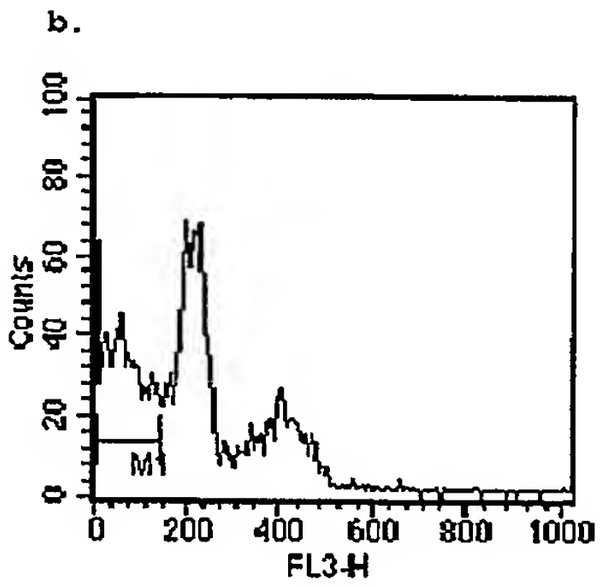


Figure 7C

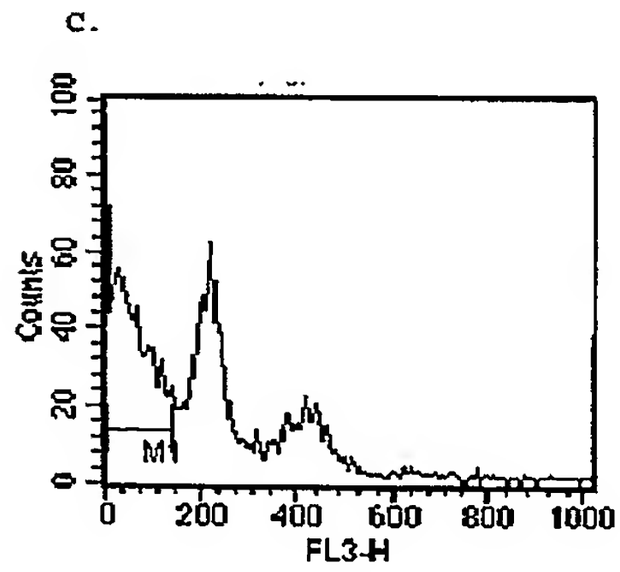


Figure 7D

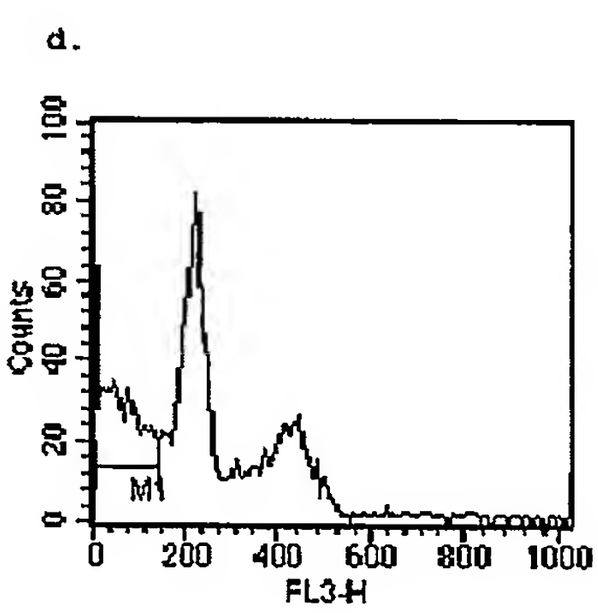


Figure 7E

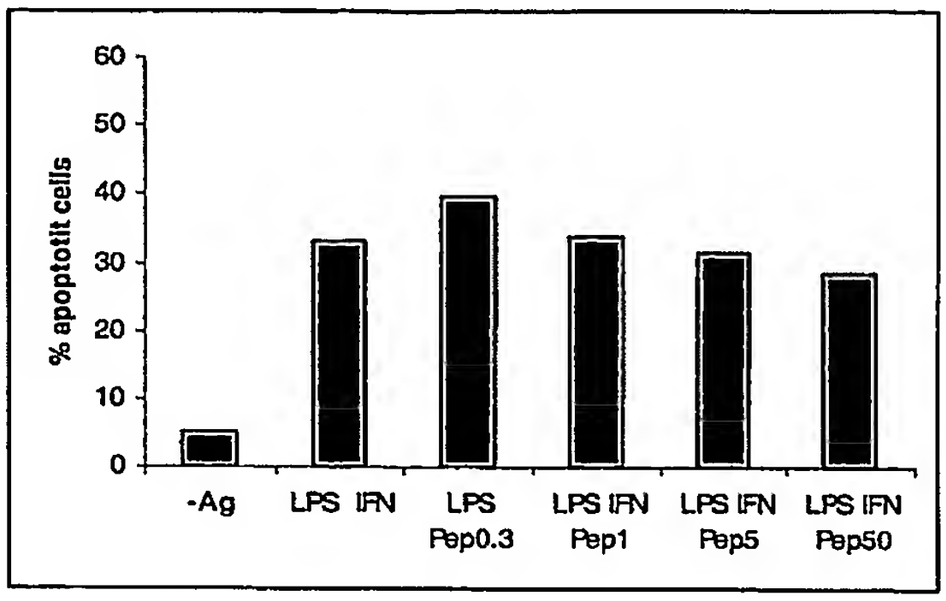


Figure 8A

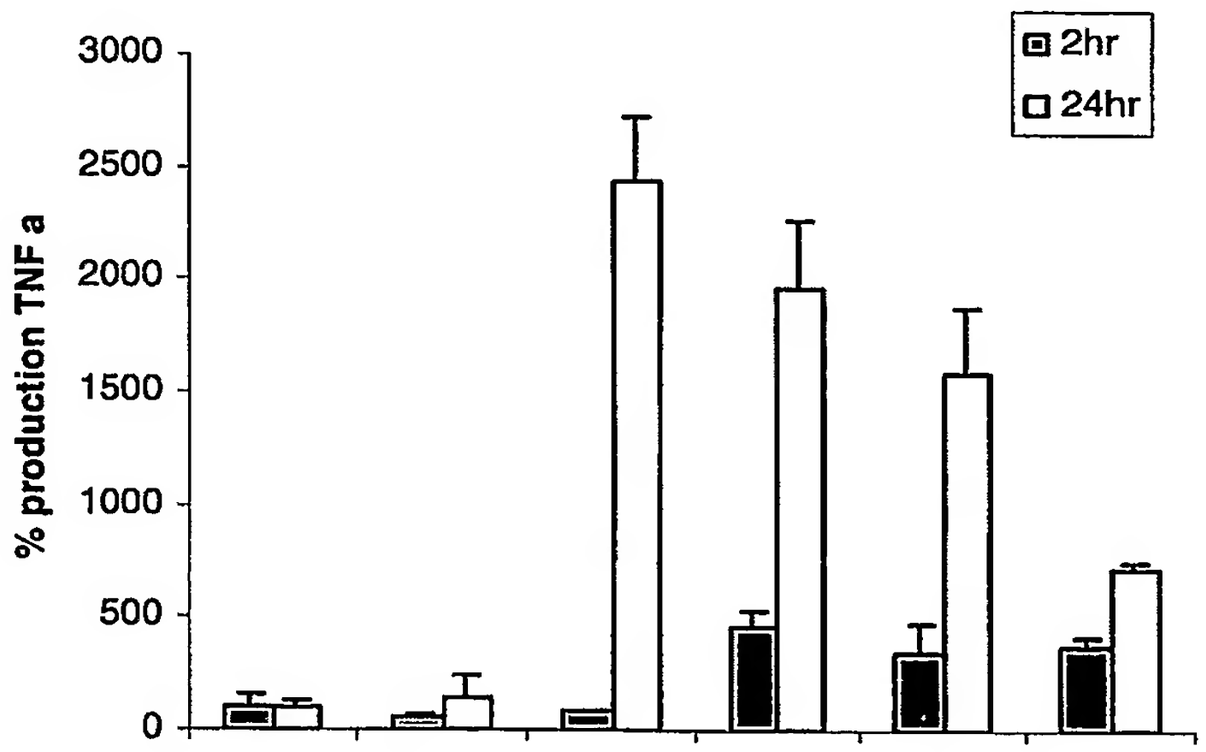
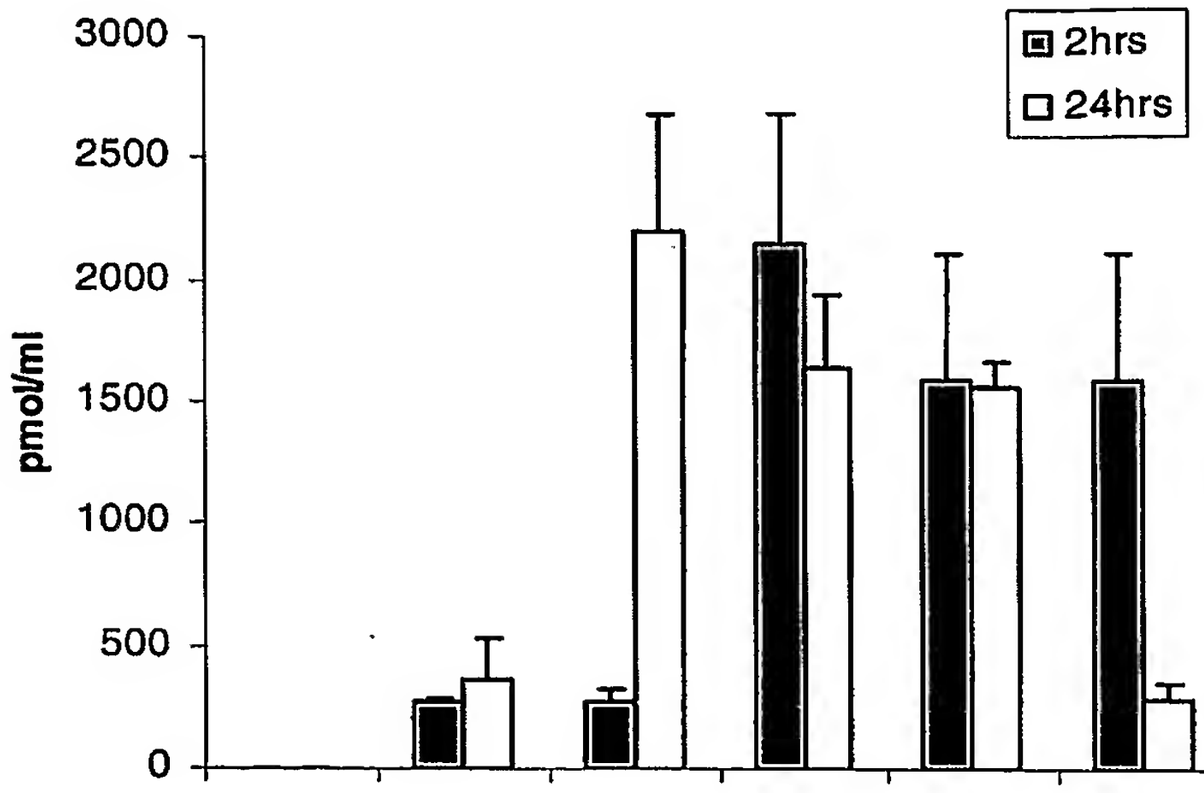
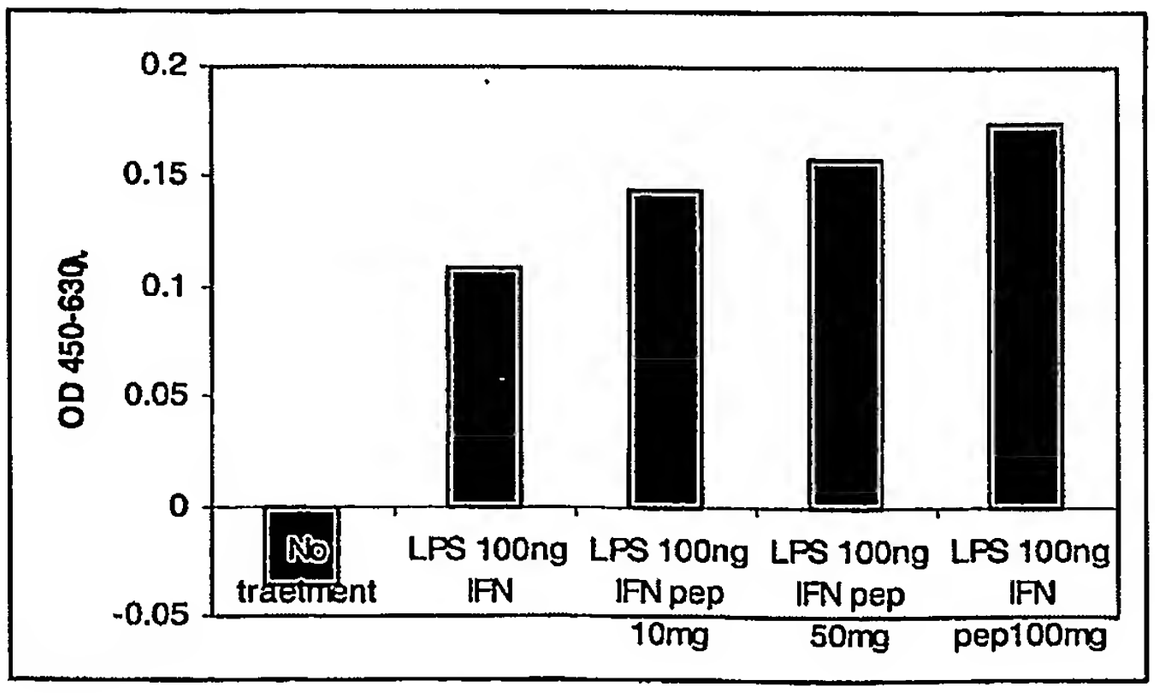


Figure 8B



LPS+IFNγ	-	+	+	+	+	+
peptide	-	-	0.3μg	10μg	50μg	-
ManLAM	-	-	-	-	-	0.1μg

Figure 8C



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